



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. **03-40155-US**

Inventors: Ho et al.

Group Art Unit No.: 1621

Application No.: 10/652,813

Examiner: Witherspoon, Sikarl A.

Filed: August 29, 2003

For: Benzotropolone Derivatives And Modulation Of Inflammatory Response

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER RULE 132

As below named coinventors of the subject matter claimed in the above-identified application, we do hereby declare that:

1. We along with Robert T. Rosen, who is unable to sign this declaration as he is presently incapacitated and is in an intensive care unit, are coauthors of the research publication in Tetrahedron Letters, 2002, 43:7129-7133.
2. The other authors of the publication are Shiyong Tian, Ruth E. Stark, Xiaofeng Meng and Chung S. Yang ("other authors").
3. The other authors merely worked under our direction and were not involved in conception of the invention including the design and methods of synthesis of the benzotropolone derivatives recited in the pending claims. Therefore, the other authors are not co-inventors of the subject matter disclosed in the research publication.

By signing below, we hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information or belief are believed to be true;

and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing there upon, or any patent to which this verified statement is directed.

Chi-Tang Ho Dec. 23. 2004

Chi-Tang Ho

Date

Shengmin Sang

Shengmin Sang

Date

12/23/2004

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TPA () preparation

25 μ l test compound = 0.5 μ mol for each compound

1. TPA
 2. Theaflavin
 3. Theaflavin-3-gallate
 4. Theaflavin-3'-gallate
 5. Theaflavin-3,3'-digallate
 6. EGCG
 7. Acetone
- 10:30 AM kitchen ~ Theaflavin
10:50 AM TPA
2:50 PM make cells

1. Acetone
 2. TPA
 3. Theaflavin (0.5 μ mol)
 4. Theaflavin-3-gallate (0.5 μ mol)
 5. Theaflavin-3'-gallate (0.5 μ mol)
 6. Theaflavin-3,3'-digallate (0.5 μ mol)
 7. EGCG (0.5 μ mol)
- OD + % cell survival at
age of 23 days old
30 days old at

The compound in 20 μ l acetone was applied topically
on both sides 20 min before TPA application.

* Inhibition

1	74.4/10 ears	7.44	0	-	7.44 \pm 0.18 (0.08)	P = 0.08
2	117.0/10 ears	11.70	4.26	-	11.70 \pm 3.05 (1.38)	
3	79.6/10 ears	7.96	0.46	89.2%	7.90 \pm 0.95 (0.42)	P = 0.015
4	78.6/10 ears	7.86	0.36	91.5%	7.86 \pm 0.53 (0.24)	P = 0.013
5	74.4/10 ears	7.44	0	100.0%	7.44 \pm 0.40 (0.18)	P = 0.008
6	70.3/10 ears	7.03	-0.41	100.0%	7.03 \pm 0.40 (0.18)	P = 0.03
7	87.9/10 ears	8.79	1.35	68.3%	8.79 \pm 1.20 (0.54)	P = 0.043

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TPA - induced edema

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		A single treatment	
1. Acetone	74.4/10 ems + 198.	= 272.4	ug
2. TPA	117.0/10 ems + 341.6	= 458.6	"
3. TPA + theoflavin	79.6/10 ems + 305.5	= 385.1	"
4. TPA + theoflavin-3-G	78.6/10 ems + 304.1	= 382.7	"
5. TPA + theoflavin-3'-G	74.4/10 ems + 347.6	= 422.0	"
6. TPA + theoflavin-3'-di-G	70.3/10 ems + 297.9	= 268.2	ug
7. EGCG	pool 2 runs weight.		
1. Acetone	14.6 (7.34), 15.1 (7.55), 14.4 (7.20), 15.1 (7.55), 15.4 (7.70)		
2. TPA	27.0 (13.5), 33.5 (16.75), 17.4 (8.7), 19.2 (9.6), 18.6 (9.3)		
3. TPA + theoflavin	19.3 (9.65), 14.3 (7.15), 15.4 (7.70), 14.1 (7.05), 16.7 (8.35)		
4. TPA + theoflavin-3-G	17.6 (8.8), 14.8 (7.4), 15.4 (7.70), 14.7 (7.35), 16.1 (8.05)		
5. TPA + theoflavin-3'-G	15.4 (7.70), 14.8 (7.4), 15.9 (7.95), 13.6 (6.8), 14.1 (7.05)		
6. TPA + theoflavin-3'-di-G	13.8 (6.90), 15.7 (7.85), 13.9 (6.95), 14.1 (7.05), 13.4 (6.70)		
7. EGCG	17.7 (8.85), 22.1 (11.05), 16.5 (8.25), 15.0 (7.50), 16.9 (8.45)		
		$n=5$	$\sqrt{5} = 2.236$
		$E = \frac{D}{\sqrt{5}}$	IL-6 (pg/ml)
1. Acetone	7.46 ± 0.18 (0.08)	0.88 ± 0.07 (0.05)	n=2
2. TPA + theoflavin	4.11 ± 0.57 ± 3.09 (1.38)	3.05 ± 0.05 (0.03)	2.17
3. TPA + theoflavin-3-G	0.33 ± 0.79 ± 0.95 (0.42)	2.48 ± 0.16 (0.11)	1.60 - 26.3
4. TPA + theoflavin-3'-G	0.40 ± 0.86 ± 0.53 (0.24)	1.76 ± 0.05 (0.03)	0.88 - 59.4
5. TPA + theoflavin-3'-di-G	0.74 ± 0.40 (0.18)	1.08 ± 0.03 (0.02)	0.20 - 90.8
6. TPA + theoflavin-3'-di-G	0.70 ± 0.40 (0.18)	1.52 ± 0.14 (0.10)	0.64 - 70.5
7. EGCG	1.36 ± 0.82 ± 1.20 (0.54)	2.10 ± 0.06 (0.04)	1.22 - 43.8

page 9. Theoflavin and its derivatives stock solutions
were diluted 1:1

3. 300 µl acetone + 300 µl theoflavin
4. 300 µl acetone + 300 µl theoflavin-3-G
5. 300 µl acetone + 300 µl theoflavin-3'-G
6. 300 µl acetone + 300 µl theoflavin-3'-di-G
7. 300 µl acetone + 300 µl EGCG

* 20 µl = 0.4 µmol

12.5 µl = 0.25 µmol

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10.00 = 0.20 µmol

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HR-4 (page 26)

1	Acetone + Acetone	7.09 ± 0.22 (0.11)	0	✓ inhibition
2	Acetone + TPA (0.3 μmol)	10.01 ± 1.28 (0.52)	2.92	
3	Nobelator ^{10-15 μmol} + TPA (0.3 μmol)	11.20 ± 1.18 (0.48)	4.11	↑ 40.8%
4	Nobelator (0.5 μmol) + TPA (0.3 μmol)	8.94 ± 0.64 (0.26)	1.85	36.6%
5	Dimenacetate (0.25 μmol) + TPA (0.3 μmol)	8.69 ± 1.23 (0.50)	1.60	45.2%
6	Dimenacetate (0.50 μmol) + TPA (0.3 μmol)	7.99 ± 0.29 (0.12)	0.90	69.2%
7	Glycolic acid (0.5 μmol) + TPA (0.3 μmol)	9.85 ± 1.53 (0.47)	2.76	5.5%
8	Glycolic acid (2.0 μmol) + TPA (0.3 μmol)	11.84 ± 0.94 (0.38)	4.75	↑ 12.7%

Female C57BL/6J (28-30 days old; 6 mice/group) were treated topically with 20 μl acetone or test compound in 20 μl acetone 20 μl topical application of TPA (0.3 μmol) five a day for 3 days and half. The mice were killed at 5 hr after the last dose of TPA treatment and punches were taken and weighed. data are expressed as the mean ± s.e.

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HR-5 Time Course of TPA - Induced Inflammation and Cytokine Protein Expression

TPA (1.6 μ M)

1	0	No TPA	(Tuesday, killed)
2	3	1:30 p.m. \rightarrow 4:30 p.m.	(Tuesday)
3	5	11:35 p.m. \rightarrow 4:35 p.m.	(Tuesday)
4	8	8:40 p.m. \rightarrow 4:40 p.m.	(Tuesday)
5	16	5:10 p.m. \rightarrow 9:10 a.m.	(Wednesday)
6	24	9:10 a.m. \rightarrow 9:10 a.m.	(Wed \rightarrow Thurs)
7	48	9:10 \rightarrow 9:10 a.m.	(Tuesday \rightarrow Thursday)
8	72	9:15 \rightarrow 9:35 a.m.	(Monday \rightarrow Thursday)

(5 mice per group)

Female CD-1 mice (9-10 weeks old) were treated topically with TPA (1.6 μ M) in 20 μ l acetone once. The mice were killed at 0, 3, 5, 8, 16, 24, 48 and 72 hours after TPA treatment. Ear punches were taken and weighed (both ears). All ear samples were stored in a -80°C for cytokine assay.

1	0 hrs:	17.1 (8.55), 17.5 (8.75), 16.2 (8.10), 17.1 (8.55), 16.2 (8.10)	
		total 10 ears, 86.3	other total 28.80
2	3 hrs:	16.8 (8.40), 14.9 (7.45), 16.9 (8.45), 14.3 (7.15), 17.4 (8.70)	
		total 10 ears, 80.8	total other 38.20
3	5 hrs:	28.2 (14.1), 21.0 (10.5), 26.4 (13.2), 27.8 (13.9), 31.8 (15.9)	
		total 10 ears, 135.6	total other 53.53
4	8 hrs:	37.4 (18.7), 28.5 (14.25), 32.9 (16.45), 36.7 (18.35), 36.9 (18.45)	
		total 10 ears, 175.8	total other 69.31
5	16 hrs:	24.3 (12.15), 25.1 (12.55), 32.0 (16.00), 19.3 (9.65), 28.1 (14.05)	
		total 10 ears, 138.9	others 47.74
6	24 hrs:	22.5 (11.25), 19.1 (9.55), 22.9 (11.45), 17.8 (8.9), 24.8 (12.4)	
		total 10 ears, 107.8	others 45.12
7	48 hrs:	16.3 (8.15), 16.2 (8.10), 17.5 (8.75), 16.7 (8.35), 15.9 (8.45)	
		total 10 ears, 82.7	other 36.76
8	72 hrs:	18.9 (9.45), 17.6 (8.80), 17.5 (8.75), 18.3 (9.15), 18.6 (9.30)	
		total 90.7	others 35.75

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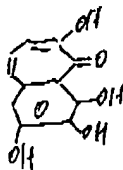
hrs after TPA

1	0	8.41 ± 0.26	(0.12)	(8.63)	100%
2	3	8.03 ± 0.61	(0.27)	(8.08)	95.5%
3	5	13.52 ± 1.75	(0.78)	(13.56)	160.8%
4	8	17.24 ± 1.69	(0.76)	(17.58)	205%
5	16	12.88 ± 2.11	(0.94)	(13.19)	153.2%
6	24	10.71 ± 1.29	(0.58)	(10.78)	127.3%
7	48	8.36 ± 0.23	(0.10)	(8.29)	99.4%
8	72	9.09 ± 0.27	(0.12)	(9.07)	108.1%

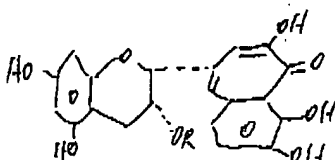
TPA (1.6 nmol) bath ears

See pages 38 and 39

11. GaCa: F.W. 220, solvent, Acetone, Pyridone. Amount: 64 mg



12, and 13



12: EGCGa; R=H, F.W. 384, solvent, Acetone, Pyridone. Amount: 51

13: EGCGa; R=gallate, F.W. 536, solvent: Acetone, Pyridone, Amount: 51

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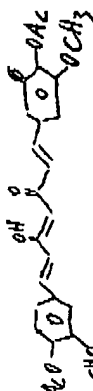
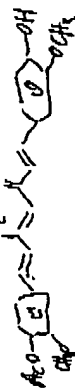
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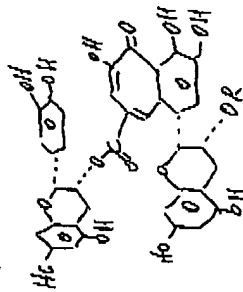
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Total: 13 Samples

FYM: SHENGEN SANG

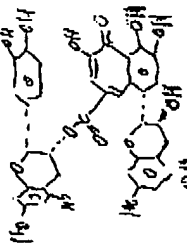
1. Cu-1: F.W.: 452, solvent: CHCl₃, Acetone, Amount: 50mg2. Cu-2: F.W.: 452, solvent: CHCl₃, Acetone, Amount: 50mg

3 and 4:

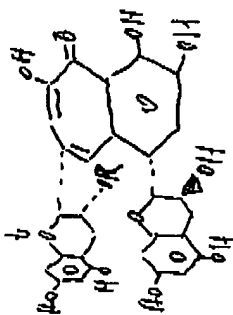
3: ~~ECG~~ ECGd; R = R gallate, F.W.: 552, solvent: Acetone, MeOH, Amount: 50mg

4: EECG; R = H, F.W.: 700, solvent: Acetone, MeOH, Amount: 50mg

5: EECG; F.W.: 700, solvent: Acetone, MeOH, Amount: 50mg

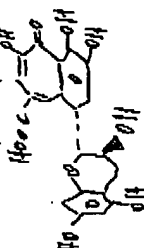


6. EECG; R = H, F.W.: 564, solvent: Acetone, MeOH, Amount: 53mg

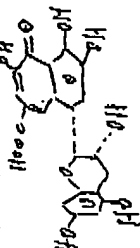


7. EECG; R = gallate, F.W.: 716, solvent: Acetone, MeOH, Amount: 51mg

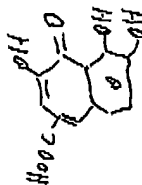
8. CGA; F.W.: 428, solvent: Acetone, MeOH, Amount: 43mg



9. EECG; F.W.: 428, solvent: Acetone, MeOH, Amount: 51mg



10. ECG; F.W.: 248, solvent: Acetone, MeOH, Amount: 61mg



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Name	mole wt	Amount	20 μ l = 0.5 μ mol	20 μ l = 1 μ mol
1. Cu-1	452	50 mg	11.3 mg/ml	4.42 ml
2. Cu-2	452	50 mg	11.3 mg/ml	2.21 ml
3. EC6di	852	50 mg	21.3 mg/ml	2.21 ml
4. ECECG	700	50 mg	17.5 mg/ml	1.175 ml
5. CECG	700	50 mg	17.5 mg/ml	1.243 ml
6. CEGC	564	53 mg	14.1 mg/ml	1.429 ml
7. CEGCG	716	51 mg	17.9 mg/ml	1.879 ml
8. CGA	428	43 mg	10.7 mg/ml	1.425 ml
9. ECGA	428	51 mg	10.7 mg/ml	2.01 ml
10. GACA	248	61 mg	6.2 mg/ml	2.335 ml
11. GACA	220	64 mg	5.5 mg/ml	4.919 ml
12. ECGCA	384	51 mg	12.75 mg/ml	5.815 ml
13. ECGCA	536	51 mg	13.4 mg/ml	2.00 ml
14. Caxcum	368	51 mg	9.2 mg/ml	1.903 ml
# 10	20 μ l = 2.0 μ mol		2.455 ml	
	= 1.0 μ mol		4.919 ml	
	= 0.5 μ mol		9.838 ml	
# 11	20 μ l = 2.0 μ mol		11.63 ml	2.908 ml
	= 1.0 μ mol		5.815 ml	
	= 0.5 μ mol		11.63 ml	
1-7	20 μ l = 1 μ mol			
8-13	20 μ l = 0.5 μ mol			

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Acetylation and Methylation of Curcumin and EGCG

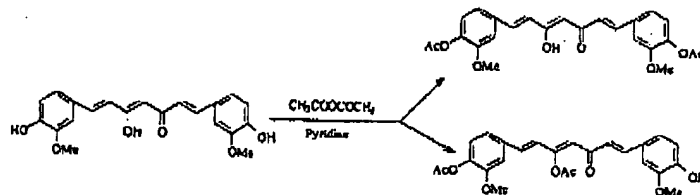
Rational and Objectives

Curcumin and EGCG, the know component in turmeric and tea, respectively, are important chemopreventive agents. It is know that these two compounds have poor bioavailability due to their high polarity. We will synthesize the acetylated and methylated derivatives of curcumin and EGCG in the hope to improve their bioavailability. 100-200 mg samples of each derivative of curcumin and EGCG will first be prepared to give to Drs. M. T. Huang for bioactivity study and also to Dr. C.S. Yang for bioactivity study.

1. Acetylation of Curcumin

We have successfully synthesized two diacetylated isomers of curcumin. 500 mg of curcumin was reacted with 0.3 mL acetyl anhydride in pyridine (3 mL) at room temperature for 6 hr. After evaporation of the solvent *in vacuo*, the residue was applied to silica gel column eluted with hexane-chloroform-methanol (2.5:2:0.2) solvent system to give 120 mg compound 1, 200 mg compound 2, and 200 mg curcumin. Both compound 1 and 2 are diacetylated isomers of curcumin. The structures of these two compounds have been established by mass spectrometry and NMR spectrometry.

We will synthesize the mono- and tri- acetylated curcumin by controlling the amount of acetyl anhydride, reaction time, and basicity of solvent.



2. Acetylation of EGCG

Using the same method we used for the preparation of the acetylated curcumin, we will be able to synthesize different acetylated EGCG. We will use Sephadex LH-20 and RP C-18 to isolate these reaction products. The structure of synthesized compounds will be identified by mass spectrometry and NMR spectrometry.

3. Methylation of EGCG

EGCG will be mixed with methyl iodide and K_2CO_3 in aqueous acetone. We will control the degree of methylation in EGCG by changing the ratio of EGCG and methyl iodide, and also the reaction time. The products will be isolated by the combination of Sephadex LH-20 column and RP C-18 column chromatography. This method has been successfully used by Dr. Meng as described in his thesis (Meng, X., Ph.D. Thesis, Rutgers University, 2002).

4. Methylation of Curcumin

The method for the methylation of EGCG will be applied to the methylation of curcumin. Again, the synthesized derivatives will be structurally identified by mass spectrometry and NMR spectrometry.

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EXP. HR-6

Read of 1st Group (page 22, Group 3-8) on

TPA + Dexamethasone and corticosteroid synthesis

TPA and Dexamethasone

1. Acetone + Acetone
2. Acetone + TPA (1 mmol)
3. Acetone + TPA (1 mmol)
4. Acetone + TPA (1 mmol)
5. Acetone + TPA (1 mmol)
6. Acetone + TPA (1 mmol)
7. Acetone + TPA (1 mmol)
8. Acetone + TPA (1 mmol)

TPA 11 (1 mmol)

Read of group 3-8 on 1st group (page 22, Group 3-8) on

5 mice per group (Female CD-1, 5 weeks old)

The mice were treated topically with 20 µl acetone or

TPA (1 mmol) in 20 µl acetone. The mice were

killed by cervical dislocation 5 hours later. The

livers were removed and weighed. The

liver weights were recorded. The

liver weights were recorded. The

1	74.7 mg	14.5 (2.15)	15.2 (7.88)	14.9 (7.45)	14.6 (7.32)	15.2 (7.60)
2	142.5 mg	33.2 (11.55)	32.4 (15.2)	28.3 (14.15)	32.2 (16.05)	35.0 (17.5)
3	83.5 mg	17.0 (8.50)	15.6 (7.80)	18.0 (9.00)	18.15 (9.15)	14.6 (7.30)
4	86.0 mg	15.7 (7.85)	17.2 (8.60)	15.7 (7.85)	18.5 (9.25)	18.4 (9.05)
5	87.3 mg	15.7 (7.85)	19.7 (9.85)	17.4 (8.70)	22.8 (11.40)	14.9 (7.45)
6	10.6 mg	16.1 (8.05)	26.4 (13.20)	20.9 (10.45)	21.1 (10.55)	21.4 (10.70)
7	85.2 mg	15.5 (7.75)	19.9 (9.95)	14.7 (7.35)	19.5 (9.75)	18.6 (9.30)
8	93.4 mg	17.3 (8.65)	17.8 (8.90)	17.7 (8.85)	16.8 (8.40)	21.2 (10.60)
adipose weight		173.6 mg	21.3 mg	31.2 mg	24.5 mg	28.6 mg
		52.3 mg	6.3 mg	17.1 mg	25.7 mg	30.4 mg

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Treatment	Average weight of ear punches (mg) (Mean ± SD)	Percent inhibition
1. Acetone + Acetone	7.44 ± 0.17	
2. Acetone + TPA (1 mmol)	15.91 ± 0.51	
3. ECEG (0.5 µmol) + TPA (1 mmol)	8.35 ± 0.71 (0.32)	89.2%
4. ECEG (0.5 µmol) + TPA (1 mmol)	8.52 ± 0.59 (0.24)	87.2%
5. ECEG (0.5 µmol) + TPA (1 mmol)	8.65 ± 1.43 (0.64)	85.7%
6. ECEG (0.5 µmol) + TPA (1 mmol)	10.58 ± 1.63 (0.73)	82.9%
7. ECEG (0.5 µmol) + TPA (1 mmol)	8.42 ± 0.93 (0.42)	88.4%
8. ECEG (0.5 µmol) + TPA (1 mmol)	9.28 ± 0.82 (0.37)	87.3%

Inhibitory Effect of Theaflavin's Derivatives
on 12-O-Tetradecanoylphorbol-13 acetate (TPA)-induced
Edema of Mouse Ear

Treatment	Average weight of ear punches (mg) (Mean ± SD)	Percent inhibition
6. Acetone + Acetone	7.44 ± 0.17	
2. Acetone + TPA (1 mmol)	15.91 ± 0.51	
3. ECEG (0.5 µmol) + TPA (1 mmol)	8.35 ± 0.71*	89.2%
4. ECEG (0.5 µmol) + TPA (1 mmol)	8.52 ± 0.59*	87.2%
5. ECEG (0.5 µmol) + TPA (1 mmol)	8.65 ± 1.43*	85.7%
6. ECEG (0.5 µmol) + TPA (1 mmol)	10.58 ± 1.63*	82.9%
7. ECEG (0.5 µmol) + TPA (1 mmol)	8.42 ± 0.93*	88.4%
8. ECEG (0.5 µmol) + TPA (1 mmol)	9.28 ± 0.82*	87.3%

Each ear of female CD-1 mice (5 mice per group; 35 days old) were treated topically with 20 µl acetone, or test compound in 20 µl acetone at 20 min prior to topical treatment of 20 µl acetone or TPA (1 mmol) in 20 µl acetone. Five hours later, the mice were killed by cervical dislocation and ear punches (6-mm in diameter) were taken and weighed.

*Statistically different from the second TPA treated group (P < 0.05) as determined by the Student's t test.

1. Ethyl acetate fraction (Cyanobacteria)
1. Ethyl acetate fraction (Cyanobacteria)
(2) Butanol fraction (Cyanobacteria)
(2) Butanol fraction (Cyanobacteria)
(3) Ethanol fraction (Cyanobacteria)
(3) Ethanol fraction (Cyanobacteria)
Caf 110: 2045, Caf 110: 2137, Shred: 20°C
9.12, 11.62 - 6 Octadecanoylphorbol-13 acetate (TPA) 25°C
10.12, 11.62 - 6 Octadecanoylphorbol-13 acetate (TPA) 25°C
Caf 110: 2045, Caf 110: 2137, Shred: 20°C

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M-T Huang

Notebook No. _____

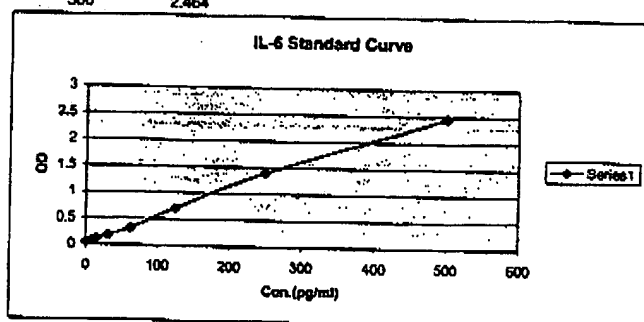
IL-6 Standard Curve
con.(pg/ml) OD

0	0.051
7.8	0.077
15.6	0.123
31.2	0.188
62.5	0.327
125	0.71
250	1.397
500	2.464

- 1 tissue con. 100mg/ml
- 2 Dilution (1:8)
- 3 100ul sample for assay.
- 4 OD used wavelength 450nm.

Exp HR-5

Page 29-30



HR-5 Time Course of TPA Induced Inflammation (1:8)

# of group	Group	OD	Con. (pg/ml)			
1	0 hour (no TPA)	0.107	13.57	13.57	13.57	0.071 1.07 ± 0
2	3 hours (TPA)	0.172	28.55	28.55	28.55	1.71
3	5 hours (TPA)	0.172	28.55	28.55	28.55	1.71 ± 0
4	8 hours (TPA)	0.437	83.52	87.345	55.14	5.47
5	16 hours (TPA)	0.477	91.17	95.685	35.54	5.24 ± 0.23 (0.16)
6	24 hours (TPA)	0.547	86.3	95.685	22.83	5.70
7	36 hours (TPA)	0.294	58.19	55.14	7.95	5.78 5.74 ± 0.04 (0.03)
8	48 hours (TPA)	0.283	54.09		3.27	3.31 ± 0.07 (0.05)
9	60 hours (TPA)	0.176	29.21	35.535	1.75	2.13 ± 0.58 (0.27)
10	72 hours (TPA)	0.178	29.54	22.825	1.77	1.37 ± 0.40 (0.28)
11	84 hours (TPA)	0.127	16.11		0.77	0.48 ± 0 (0)
12	96 hours (TPA)	0.079	8	7.95	0.47	
13	108 hours (TPA)	0.078	7.9		0.47	

16.67%

Dr. Bob Rosen,

W: Bioassay results?

1

Delivered-To: mhuang@aesop.rutgers.edu
 Date: Mon, 1
 From: "Dr. Bob Rosen" <rosen@AESOP.RUTGERS.EDU>
 Subject: FW: Bioassay results?
 To: mhuang@aesop.rutgers.edu
 Cc: vorsa@AESOP.RUTGERS.EDU
 Reply-to: rosen@AESOP.RUTGERS.EDU
 Importance: Normal
 X-Priority: 3 (Normal)
 Status:

MT

Chi-Tang sent over some samples that Nick Vorsa extracted. These were from blueberry and/or cranberry.

Did you have a chance to do a mouse ear test yet to see which extract best reduced inflammation?

Nick needs any simple assay to see which extract is best to continue with his isolations

Thanks

Bob Rosen

-----Original Message-----

From: Nichol Vorsa [mailto:vorsa@AESOP.RUTGERS.EDU]
 Sent: Wednesday,
 To: Dr. Bob Rosen
 Subject: Bioassay results?

Bob:

I hope you are having a good summer. Iina and I are wondering how the bioassay(s) went and when might the results be available?

regards,

Nick

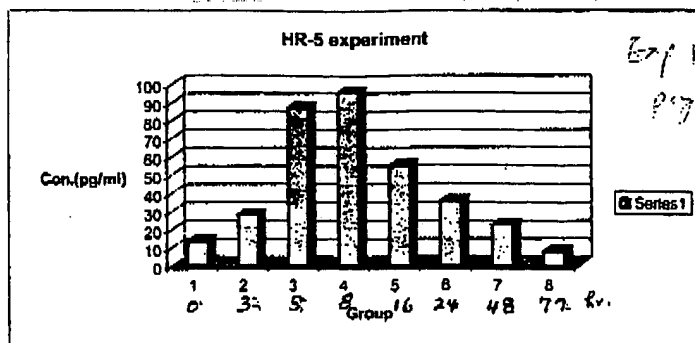
Continued on Page

By

Huang

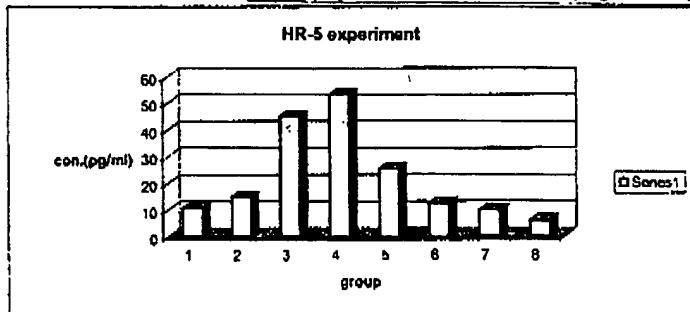
AC

Date



HR-5 Time Course of TPA Induced Inflammation (1:12) 8-33-2016 n=4 P8/mg

# of group	Group	OD	Con. (pg/ml)	Mean	SD	SE	t-value	p-value
0	1 0 hour (no TPA)	0.12	15.22	10.8	0.81	10.8	1.83	1.14 ± 0.49 (0.30)
3	2 3 hours (TPA)	0.083	6.38	15.03	1.71	45.06	1.83	1.14 ± 0.49 (0.30)
5	3 4 hours (TPA)	0.121	15.22	15.03	1.71	45.06	1.83	1.14 ± 0.49 (0.30)
8	4 5 hours (TPA)	0.117	14.84	15.03	1.71	45.06	1.83	1.14 ± 0.49 (0.30)
16	5 6 hours (TPA)	0.265	43.98	45.06	5.01	25.73	5.28	5.33 ± 0.21 (0.11)
24	6 7 hours (TPA)	0.278	46.14	45.06	5.01	25.73	5.28	5.33 ± 0.21 (0.11)
48	7 8 hours (TPA)	0.289	55.24	53.425	5.78	10.13	6.63	6.08 ± 0.37 (0.14)
72	8 9 hours (TPA)	0.27	51.61	53.425	5.78	10.13	6.63	6.08 ± 0.37 (0.14)
		0.182	26.39	25.725	3.57		3.17	3.20 ± 0.13 (0.07)
		0.151	25.06		3.24		3.01	3.20 ± 0.13 (0.07)
		0.078	7.8	12.64	1.75		0.95	1.83 ± 0.57 (0.29)
		0.137	17.38		2.51		2.09	1.83 ± 0.57 (0.29)
		0.084	9.52	10.13	1.77		1.14	1.29 ± 0.70 (0.15)
		0.106	10.74		0.97		1.29	1.29 ± 0.70 (0.15)
		0.087	6.79	6.13	0.44		0.82	0.61 ± 0.14 (0.07)
		0.054	5.47		0.47		0.46	0.61 ± 0.14 (0.07)



Handwritten signature/initials.

40
PR

- 1 Issue con. 100mg/ml
2 Dilution (1:5, 1:10)
3 50ul sample for assay
4 OD used wavelength 450nm

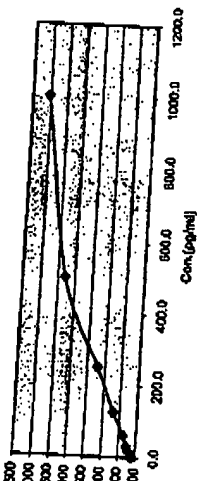
Exp HA-5

page 29-30

N-18 Standard Curve

Con. (pg/ml)	OD
0.0	0.121
15.6	0.188
31.2	0.272
62.5	0.371
125.0	0.689
250.0	1.209
500.0	2.287
1000	3.028

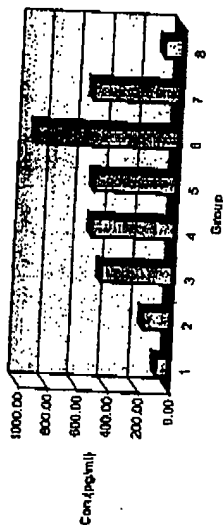
N-18 Standard Curve



HR-5 Time Course of TPA Induced Inflammation (1:5) 2004/4

# of group	10 hours (no TPA)	OD	Con. (pg/ml)
1	0.451	75.98	78.74
2	0.46	77.49	78.735
3	0.98	171.67	172.475
4	0.988	173.28	172.48
5	2.127	465.02	459.01
6	2.072	453	459.01
7	2.48	542.2	532.36
8	2.39	522.52	530.83
9	2.391	522.74	530.83
10	2.485	538.92	534.465
11	2.823	931.98	934.465
12	2.838	936.94	934.465
13	2.568	561	561
14	2.589	561	561
15	0.837	113.81	114.985
16	0.648	118.06	118.06
17	0.107	0	0
18	0.097	0	0

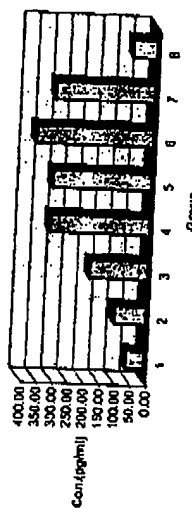
HR-5 experiment



HR-5 Time Course of TPA Induced Inflammation (1:10)

# of group	10 hours (no TPA)	OD	Con. (pg/ml)
1	0.372	82.67	59.47
2	0.334	58.27	59.47
3	0.575	102.83	102.83
4	0.575	102.83	102.83
5	1.092	118.09	185.39
6	1.222	252.89	314.62
7	1.472	304.38	318.985
8	1.594	329.61	315.55
9	1.543	319.07	314.62
10	1.5	310.17	314.62
11	1.737	378.76	371.885
12	1.655	394.01	371.885
13	1.473	304.59	315.55
14	1.579	326.51	326.51
15	0.43	72.44	74.965
16	0.46	77.49	74.965
17	0.118	0	0
18	0.125	0	0

HR-5 Experiment



M-T Huang

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PROJECT

Notebook No.

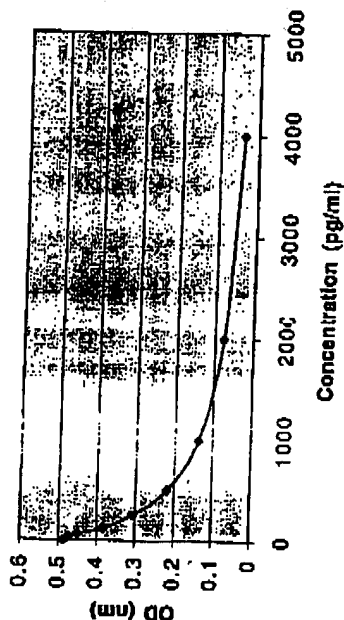
- 1) Dilution 1:100
- 2) 50 ul sample for assay
- 3) OD used wavelength 420 nm

Exp HR-5
pages 29-30

Stat-PGE2 Standard Curve

Concentration (pg/ml)	OD
0	0.4865
31.25	0.4745
62.5	0.451
125	0.3825
250	0.3065
500	0.218
1000	0.138
2000	0.078
4000	0.036

Stat-PGE2 Standard Curve



Concentration (pg/ml)	OD
1050	0.501
500	0.303
250	0.242
125	0.163
62.5	0.088
31.25	0.041
15.625	0.018

Sample (1:100 Dilution)	Concentration (pg/ml)
1) 0 hrs - No TPA	0.501
2) 3 hrs	0.303
3) 5 hrs	0.242
4) 8 hrs	0.163
5) 16 hrs	0.088
6) 24 hrs	0.041
7) 48 hrs	0.018
8) 72 hrs	0.009

Blank = 0.084

pg/ml

DATA	Raw Data	Minus Blank	Average
B0	0.556	0.472	0.4865
B1	0.585	0.501	0.4745
F1	0.534	0.47	0.451
F2	0.563	0.478	0.3825
F3	0.512	0.428	0.3065
F4	0.558	0.474	0.218
F5	0.433	0.369	0.138
F6	0.48	0.399	0.078
F7	0.385	0.301	0.036
F8	0.396	0.312	0.218
F9	0.297	0.213	0.138
F10	0.307	0.223	0.076
F11	0.213	0.129	0.038
F12	0.231	0.147	0.501
F13	0.184	0.08	0.133
F14	0.156	0.072	0.127
F15	0.121	0.037	0.131
F16	0.118	0.035	0.218
F17	0.562	0.478	0.242
F18	0.605	0.521	0.303
F19	0.598	0.504	0.282
F20	0.228	0.142	0.287
F21	0.211	0.127	0.174
F22	0.215	0.131	0.156
F23	0.312	0.228	0.174
F24	0.302	0.218	0.32
F25	0.289	0.202	0.404
F26	0.325	0.241	0.318
F27	0.318	0.234	0.338
F28	0.404	0.32	0.404
F29	0.376	0.292	0.376
F30	0.381	0.287	0.258
F31	0.258	0.174	0.244
F32	0.24	0.156	0.277
F33	0.244	0.158	0.344
F34	0.277	0.193	0.248
F35	0.344	0.28	0.23
F36	0.248	0.164	0.225
F37	0.23	0.146	0.221
F38	0.23	0.141	0.236
F39	0.225	0.141	0.228
F40	0.221	0.137	0.242
F41	0.236	0.152	0.242
F42	0.236	0.152	0.242
F43	0.228	0.144	0.242
F44	0.242	0.158	0.242

1:100 Dilution

Concentration (pg/ml)

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Date

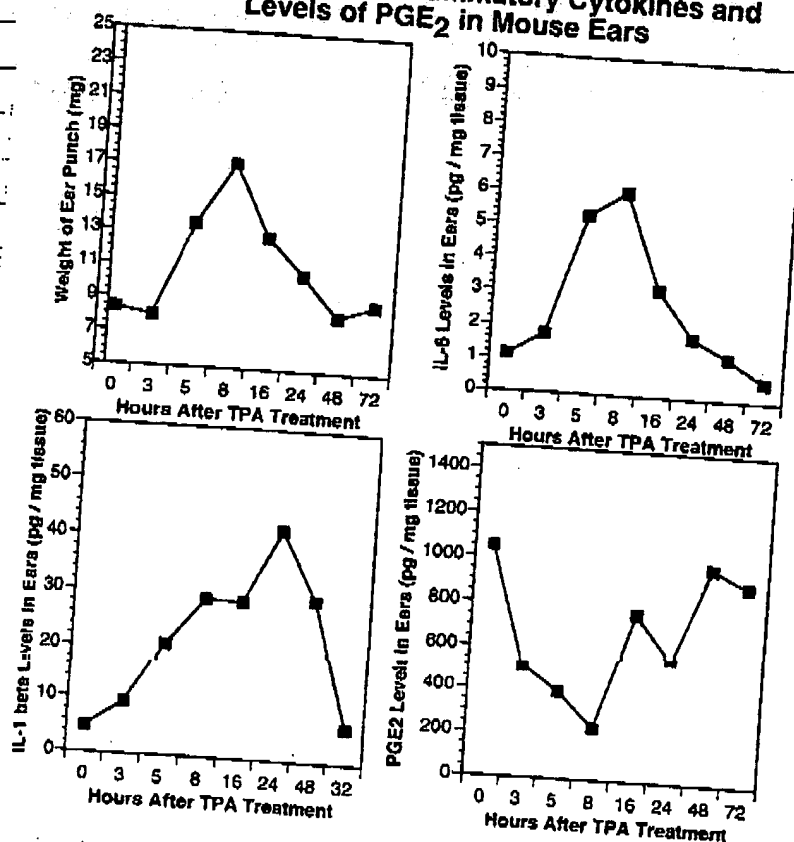
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PROJECTok No. _____
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HR-5 (p 29 + 30)

Time Course of TPA-induced Ear Edema,
Production of Pro-inflammatory Cytokines and
Levels of PGE₂ in Mouse EarsExp HR-5 Time course of TPA-induce ear edema and increases in
expression of pro-inflammatory cytokine genes in CD-1 mice

Hours after TPA	IL-1 beta (pg / mg)	IL-6 (pg / mg)	TNF alpha (pg / mg)	PGE ₂ (pg / mg)	LTB ₄ (pg / mg)	Edema (mg/punch)
0	4.89±0.54	1.14±0.30		1053.33±23.73	10.30	8.41±0.12
3	9.45±0.42	1.76±0.03		506.67±19.05	8.45	8.03±0.27
5	20.75±2.62	5.33±0.11		396.67±11.86	8.30	13.50±0.78
8	29.16±1.36	6.08±0.14		236.67±15.15	6.25	17.24±0.76
16	29.01±0.96	3.20±0.07		773.33±62.78	8.15	12.88±0.94
24	41.96±2.40	1.83±0.29		565.00±100.19	14.00	10.71±0.58
48	29.80±0.96	1.29±0.15		990.00±4.71	16.67	8.36±0.10
72	6.49±0.37	0.61±0.07		913.33±33.45	8.45	9.09±0.12

Female CD-1 mice (9-10 weeks old; 5 mice per group) were treated topically with acetone or TPA (1.6 nmol) in 20 µl acetone. The mice were killed by cervical dislocation At 3, 5, 8, 16, 24, 48, and 48 hours after TPA treatment. Ear punches (6 mm- in diameter) were taken and weighed. The ear samples were stored in a - 80 °C freezer for cytokine assays.

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PROJECT

AR-9 (Effect of Black Tea Combined with TPA)

on TPA study of mouse ear edema

1. Acetone

2. TPA

3. Test comp 9

4. Test comp 11

5. Test comp 12

6. Test comp 13

7. theaflavin

		mean \pm SE	% Inhibition
1.	Acetone (20 μ l)	7.17 \pm 0.41 (0.18)	- $p = 4.4 \times 10^{-6}$
2.	TPA (1 mmol) = 20 μ l	15.85 \pm 1.91 (0.8%)	-
3.	C8 (20 μ l = 0.5 mmol) + TPA (1 mmol)	8.95 \pm 0.97 (0.43)	79.5% $p = 4.6 \times 10^{-5}$
4.	C9 (") + TPA (1 mmol)	9.39 \pm 1.39 (0.62)	74.4% $p = 1.42 \times 10^{-5}$
5.	C11 (") + TPA (1 mmol)	8.61 \pm 1.19 (0.54)	83.4% $p = 4.15 \times 10^{-5}$
6.	C12 (") + TPA (1 mmol)	7.86 \pm 0.41 (0.18)	92.1% $p = 8.23 \times 10^{-5}$
7.	C13 (") + TPA (1 mmol)	7.49 \pm 0.76 (0.12)	96.3% $p = 5.38 \times 10^{-5}$
8.	theaflavin (") + TPA (1 mmol)	8.23 \pm 0.90 (0.40)	87.8% $p = 2.05 \times 10^{-5}$

Female C10-1 (6 weeks old)

1. Acetone	13.8 (6.4), 14.5 (7.25), 14.4 (7.20), 15.2 (7.60), 14.8 (7.40)	total 10 ears, 7.12 mg/ear, other 2+3=
2. TPA	15.45 (30.9), 25.0 (42.5), 36.3 (18.15), 34.2 (19.10), 32.1 (16.05)	total 10 ears, 15.86 mg/ear, other 494 mg
3. C8	20.7 (10.35), 19.5 (9.75), 15.7 (7.85), 17.5 (8.75), 16.1 (8.05)	total 10 ears, 90.3 mg/ear, total other 402.4 mg
4. C9	17.2 (8.60), 19.7 (9.85), 23.8 (11.90), 17.3 (8.65), 15.9 (7.95)	total 10 ears, 92.2 mg/ear, other 334.7 mg
5. C11	19.0 (9.50), 13.4 (6.70), 16.4 (8.20), 22.4 (11.2), 16.9 (8.45)	total 10 ears, 8.68 mg/ear, others 367.1 mg, 371.2 mg
6. C12	16.2 (8.1), 15.0 (7.5), 15.2 (7.1), 17.1 (8.55), 15.1 (7.55)	total 10 ears, 7.87 mg/ear, other 367.1 mg
7. C13	14.3 (7.15), 15.7 (7.85), 15.3 (7.65), 14.5 (7.25), 15.1 (7.55)	total 10 ears, 7.42 mg/ear, total 305.9 mg
8. Theaflavin	16.8 (8.4), 19.0 (9.5), 14.0 (7.0), 17.6 (8.8), 14.9 (7.45)	total 10 ears, 8.43 mg/ear, 331.7 mg

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Huang
Signed

Date

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Date

**Inhibitory Effect of Theaflavin's Derivatives
on 12-O-Tetradecanoylphorbol-13 acetone (TPA)-induced
Edema of Mouse Ear**

Treatment	Average weight of ear punches (mg) (Mean \pm SE)	Percent inhibition
1. Acetone + Acetone	7.17 \pm 0.18*	-
13 Acetone + TPA (1 nmol)	15.85 \pm 0.86	-
8. CGA (0.5 μ mol) + TPA (1 nmol)	8.95 \pm 0.43*	79.5%
9. EGCGA (0.5 μ mol) + TPA (1 nmol)	9.39 \pm 0.43*	74.4%
11. gaCa (0.5 μ mol) + TPA (1 nmol)	8.61 \pm 0.54*	83.3%
12. EGCCa (0.5 μ mol) + TPA (1 nmol)	7.86 \pm 0.18*	92.1%
13. EGCGCa (0.5 μ mol) + TPA (1 nmol)	7.49 \pm 0.12*	96.3%
14 Theaflavin (0.5 μ mol) + TPA (1 nmol)	8.23 \pm 0.40*	87.8%

Both ears of female CD-1 mice (5 mice per group; 35 days old) were treated topically with 20 μ l acetone, or test compound in 20 μ l acetone at 20 min prior to topical treatment of 20 μ l acetone or TPA (1 nmol) in 20 μ l acetone. Five hours later, the mice were killed by cervical dislocation and ear punches (6-mm in diameter) were taken and weighed.

*Statistically different from the second TPA treated group ($P < 0.05$) as determined by the Student's t test.

HR-10

**Inhibitory effect of curcumin and curcumin acetate on TPA-induced
edema of mouse ear**

Treatment	Number of mice per group	Weight of ear punch (mg)	Percent inhibition
1. Acetone	5	7.61 \pm 0.35*	-
2 TPA (1 nmol)	5	11.94 \pm 0.90	-
3. Curcumin acetate-1 (0.25 μ mol) + TPA (1 nmol)	5	9.24 \pm 0.29*	62.4%
4. Curcumin acetate-1 (0.75 μ mol) + TPA (1 nmol)	5	7.39 \pm 0.20*	100.0%
5. Curcumin acetate-2 (0.25 μ mol) + TPA (1 nmol)	5	7.75 \pm 0.15*	96.8%
6. Curcumin acetate-2 (0.75 μ mol) + TPA (1 nmol)	5	7.45 \pm 0.19*	100.0%
7. Curcumin (0.25 μ mol) + TPA (1 nmol)	5	8.58 \pm 0.30*	77.6%
8. Curcumin (0.75 μ mol) + TPA (1 nmol)	5	7.92 \pm 0.30*	92.8%

Female CD-1 mice (5 weeks old; 5 mice per group) were treated topically with 20 μ l acetone or test compound in 20 μ l acetone at 20 minutes before topical application of 20 μ l acetone or TPA (1 nmol) in 20 μ l acetone. Five hours later, all mice were killed by cervical dislocation. Ear punches (6-mm in diameter) were taken and weighed. Data are expressed as the mean \pm SE.

*Statistically different from group 2 TPA alone ($P < 0.05$) determined by the Student's t test.

Signature _____
Date _____

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Date _____

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Date _____